Developmental Biology **209,** 186–199 (1999)
Article ID dbio.1999.9247, available online at http://www.idealibrary.com on IDEAL®

p82/CPEB *in Vitro*: Implications for Dual and Independent Roles of MAP and Cdc2 Kinases

Yoshinao Katsu,*,[1] Nicola Minshall,*,† Yoshitaka Nagahama,‡ and Nancy Standart*,†,[2]

**Department of Biochemistry, University of Cambridge, Cambridge CB2 1GA, England; †Marine Biological Laboratory, Woods Hole, Massachusetts 02543; and ‡Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444, Japan*

During early development gene expression is controlled principally at the translational level. Oocytes of the surf clam *Spisula solidissima* contain large stockpiles of maternal mRNAs which are translationally dormant or masked until meiotic maturation. Fertilisation of the oocyte leads to rapid polysomal recruitment of the abundant cyclin and ribonucleotide reductase mRNAs at about the time they undergo cytoplasmic polyadenylation. Clam p82, a 3′ UTR RNA-binding protein, and a member of the CPEB (cytoplasmic polyadenylation element binding protein) family, functions as a translational masking factor in oocytes and as a polyadenylation factor in fertilised eggs. In meiotically maturing clam oocytes, p82/CPEB is rapidly phosphorylated on multiple residues to a 92-kDa apparent size, prior to its degradation during the first cell cleavage. Here we examine the protein kinase(s) that phosphorylates clam p82/CPEB using a clam oocyte activation cell-free system that responds to elevated pH, mirroring the pH rise that accompanies fertilisation. We show that p82/CPEB phosphorylation requires Ca^{2+} (<100 μM) in addition to raised pH. Examination of the calcium dependency combined with the use of specific inhibitors implicates the combined and independent actions of cdc2 and MAP kinases in p82/CPEB phosphorylation. Calcium is necessary for both the activation and the maintenance of MAP kinase, whose activity is transient *in vitro*, as *in vivo*. While cdc2 kinase plays a role in the maintenance of MAP kinase activity, it is not required for the activation of MAP kinase. We propose a model of clam p82/CPEB phosphorylation in which MAP kinase initially phosphorylates clam p82/CPEB, at a minor subset of sites that does not alter its migration, and cdc2 kinase is necessary for the second wave of phosphorylation that results in the large mobility size shift of clam p82/CPEB. The possible roles of phosphorylation for the function and regulation of p82/CPEB are discussed.

Key Words: **maternal mRNA; translational activation; cytoplasmic polyadenylation; ubiquitin-mediated proteolysis.**

INTRODUCTION

Oocytes of many animals contain translationally repressed mRNAs that are activated in a stage- and sequence-specific manner during meiotic maturation and early embryogenesis (reviewed by Curtis *et al.*, 1995; Wickens *et al.*, 1996). We study the regulatory mechanisms responsible for translational activation of surf clam (*Spisula solidissima*) maternal mRNAs. Clam oocytes, arrested in prophase I, complete meiosis upon fertilisation and then proceed directly into the mitotic cell division cycles (Hunt *et al.*, 1992). During meiotic maturation, the translation of three abundant maternal mRNAs is activated, encoding cyclins A and B and the small subunit of ribonucleotide reductase (RR), whose products enable cell cycle progression and DNA synthesis (Standart *et al.*, 1992). Clam maternal mRNAs, in common with mRNAs from other organisms, undergo poly(A) tail changes during oocyte maturation, and these changes essentially correlate with translational activity (Rosenthal *et al.*, 1987). Cyclin A and RR mRNAs, masked in oocyte lysates, can be specifically translationally activated using antisense RNAs directed to the masking

[1] Present address: Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444, Japan.

[2] To whom correspondence and reprint requests should be addressed at the Department of Biochemistry, 80 Tennis Court Road, Old Addenbrookes Site, University of Cambridge, Cambridge CB2 1GA, England. Fax: +44 1223 766002. E-mail: nms@mole.bio.cam.ac.uk.

0012-1606/99 $30.00

elements located approximately in the centre of their 3′ UTRs. Importantly, in the unmasking assay, activation of translation occurred in the absence of polyadenylation, implying that the two events are not obligatorily coupled, at least *in vitro* (Standart *et al.,* 1990). An 82-kDa oocyte protein (p82) selectively binds the U-rich masking elements in the 3′ UTRs of RR and cyclin A mRNAs. p82 undergoes rapid phosphorylation in meiotically maturing clam oocytes, prior to its degradation during the first cell cleavage. Phosphorylation precedes and, according to inhibitor studies *in vitro* and *in vivo,* may be required for translational activation of maternal mRNA (Walker *et al.,* 1996, 1999).

In early development, the evolutionarily conserved mechanism of cytoplasmic polyadenylation regulates the expression of several well-characterised maternal mRNAs. The sequences that control cytoplasmic poly(A) addition during meiotic maturation include a 3′ UTR U-rich consensus motif, $U_{4\text{-}6}A_{1\text{-}2}U$, the so-called cytoplasmic polyadenylation element (CPE), proximal to the ubiquitous nuclear polyadenylation signal, AAUAAA. Both elements are required to support cytoplasmic poly(A) extension and to stimulate translation during oocyte maturation and early development in *Xenopus* and mouse (reviewed by Richter *et al.,* 1996; Wickens *et al.,* 1996). *c-mos* mRNA, whose polyadenylation and consequent translation is a pivotal regulatory step in meiotic maturation of *Xenopus* and mouse oocytes (Sheets *et al.,* 1995; Gebauer *et al.,* 1994), illustrates the importance of this modification.

The *Xenopus* CPEB protein that specifically binds the CPE motif of B4 mRNA is phosphorylated during meiotic maturation, directly or indirectly, by cdc2 kinase (Paris *et al.,* 1991). Use of antibodies to the cloned protein demonstrated the positive role of CPEB in cytoplasmic polyadenylation, initially of B4 mRNA and subsequently of c-mos, cdk-2, cyclins, and G10 mRNAs (Hake and Richter, 1994; Stebbins-Boaz *et al.,* 1996). This protein binds the CPEs of these mRNAs and is required for their polyadenylation in egg lysates (Stebbins-Boaz *et al.,* 1996). Injection of CPEB antibody into oocytes not only prevents polyadenylation *in vivo,* but also blocks progesterone-induced maturation, suggesting that CPEB is critical for early development (Stebbins-Boaz *et al.,* 1996). To promote polyadenylation, CPEB may recruit or stabilise factors such as cleavage and polyadenylation specificity factors and poly(A) polymerase to the 3′ terminus of CPE-containing mRNAs (Bilger *et al.,* 1994).

Recently, we showed that cloned clam p82 is highly homologous to *Xenopus* and mouse CPEBs (Walker *et al.,* 1999). Sequence homology is particularly striking in the C-terminal RRM didomain and the zinc finger regions, which constitute the RNA-binding portion of CPEB proteins (Hake *et al.,* 1998). We provided evidence that clam p82/CPEB plays a dual role in regulating maternal mRNA—in the oocyte it acts as a translational repressor, while in the activated egg it plays a positive role in polyadenylation (Minshall *et al.,* 1999). How can both functions of clam p82/CPEB be reconciled? Robust translational activation of masked mRNAs, triggered by fertilisation, may result from the combination of derepression and poly(A) lengthening. It is doubtless noteworthy that the two functions are temporally and cell-cycle-stage distinct and that the protein is modified, by phosphorylation, between the two stages (Walker *et al.,* 1999).

Meiotic cell division depends on the response of the oocyte to extracellular signals, including hormones (*Xenopus,* starfish) and fertilisation (*Spisula*). These signals are transduced in an unknown manner to trigger the transition to M phase, characterised by germinal vesicle breakdown (GVBD). An initial increase of Ca^{2+} influx is the primary trigger leading to GVBD in surf clam oocytes, facilitated by an increase in intracellular pH (Allen, 1953; reviewed Colas and Dubé, 1998). Two major protein kinases, cdc2 kinase and MAP kinase, are implicated in the induction of oocyte maturation. Cdc2 kinase, the catalytic subunit of MPF, in association with cyclin B, triggers all the changes that accompany oocyte maturation, such as GVBD, chromosome condensation, and spindle formation. The mechanism of MPF activation differs between species (Taieb *et al.,* 1997). In *Xenopus,* starfish, and clam oocytes, cdc2 kinase and cyclin B are already present as pre-MPF (inactive cdc2-cyclin B complex) in immature oocytes, which is activated following hormone or sperm stimulation. In contrast, immature fish oocytes contain no detectable cyclin B protein, maturation of which is synthesised *de novo* during hormone-induced oocyte. In fish, cyclin B synthesis is crucial for MPF activation (Katsu *et al.,* 1993; Nagahama *et al.,* 1995; Kondo *et al.,* 1997).

MAP kinases are serine/threonine protein kinases that are activated by various extracellular stimuli that influence cell proliferation and differentiation, such as growth factors, cytokines, and hormones, and function as key molecules in signalling processes induced by these stimuli (reviewed by Ruderman, 1993; Nishida and Gotoh, 1993; Kyriakis and Avruch, 1996). MAP kinase is activated as the result of phosphorylation on tyrosine and threonine residues by MAP kinase kinase (MAPKK or MEK). In *Xenopus* oocytes, progesterone stimulation sets off a signalling pathway culminating in the translation and stabilisation of *mos,* a MAP kinase kinase kinase. Active MAPK leads to MPF activation and oocyte maturation. MAP kinase is necessary not only for the induction of GVBD but also for the arrest at metaphase II in *Xenopus* oocytes (reviewed in Sagata, 1997). In starfish oocytes, MAP kinase activation depends on protein synthesis, like in *Xenopus* oocytes. However, MAP kinase is not required for either the activation of MPF or the induction of oocyte maturation (Sadler and Ruderman, 1998). Rather, MAP kinase in the haploid unfertilised starfish (*Asterina pectinifera and Asterina miniata*) egg is required for the suppression of DNA synthesis (Tachibana *et al.,* 1997; Sadler and Ruderman, 1998), while in *Astropecten aranciacus,* postmeiotic eggs maintain high MAP kinase activity and replicate the maternal genome, even in the absence of fertilisation (Picard *et al.,* 1996). In *Spisula,* MAP kinase is rapidly and transiently activated upon fer-

tilisation, during meiosis I (Shibuya *et al.*, 1992). Since protein synthesis in clam oocytes is not required for the completion of meiosis I (Hunt *et al.*, 1992), activation of MAP kinase does not require new polypeptide synthesis. The role of MAP kinase in MPF activation and the initiation of maturation in clam oocytes is not known.

Recently, two groups reported that cdc2 kinase plays an important role in cytoplasmic polyadenylation during *Xenopus* oocyte maturation (Ballantyne *et al.*, 1997; de Moor and Richter, 1997). Here we have continued our analysis of clam p82/CPEB phosphorylation using a cell-free activation system of clam oocytes (Walker *et al.*, 1996). We provide evidence that both cdc2 kinase and MAP kinase are necessary for hyperphosphorylation of clam p82/CPEB *in vitro*. We also show that the activation of MAP kinase is dependent on intracellular calcium and that the activations of cdc2 kinase and MAP kinases occur on independent, parallel pathways.

MATERIALS AND METHODS

Preparation of Cell-Free Extracts and pH Activation

Adult surf clams (*S. solidissima*) were provided by the Marine Biological Laboratory Marine Resource Center, Woods Hole, Massachusetts. Postmitochondrial lysates were prepared from homogenates of oocytes suspended in 0.5 vol T buffer (300 mM glycine, 100 mM Hepes, 120 mM potassium gluconate, 100 mM taurine, 40 mM NaCl, 2.5 mM $MgCl_2$, pH 6.8 with KOH); for details see Walker *et al.* (1996). Supernatants obtained after two consecutive 10-min centrifugations at 10,000*g* (Sorvall HB4 rotor) were aliquoted, frozen in liquid N_2, and stored thereafter at −80°C. Subsequently, each thawed aliquot was reclarified by centrifugation prior to use. For pH activation, clam oocyte extracts were added to an equal volume of T pH 6.8 buffer for control incubations or T pH 8.0 buffer for activation and a 1/20th volume of a 20× ATP-regenerating system (20 mM $MgCl_2$, 20 mM ATP, 200 mM creatine phosphate, and 1 mg/ml creatine phosphokinase) and incubated at 18°C. Reactions were stopped by addition of SDS sample buffer for immunoblot analysis or frozen in dry ice for measuring kinase activity later. Several batches of oocyte lysates were examined in this study.

Electrophoresis and Immunoblot Analysis

SDS–polyacrylamide gel electrophoresis was carried out using 15, 12, or 10% gels. Proteins were transferred onto Immobilon P membrane (Millipore) using a semidry blotting apparatus and treated with antibodies (Walker *et al.*, 1996). The rabbit anti-clam p82/CPEB antibody (Minshall *et al.*, 1999) was used here at a dilution of 1:25,000. The MAP kinase antibody K23 (against rat ERK1; Santa Cruz Biotechnology) was used at a dilution of 1:1000. Blots were developed using the alkaline phosphatase detection method (Harlow and Lane, 1988).

Immunoprecipitation

To immunoprecipitate p82, 40 μl of reaction mix (lysate plus buffer) was diluted with 100 μl of buffer T and, following the addition of 1 μl anti-p82 serum, incubated on ice for 1 h. Protein A–Sepharose (40 μl of 1:1 suspension; Pharmacia) was then added, and the samples were mixed for 4 h at 4°C. Following brief pelleting of beads in a microfuge, they were washed three times with 1 ml T buffer containing 0.2% Tween 20. In the experiment shown in Fig. 1A, the immunoprecipitates were subsequently washed with alkaline phosphatase buffer (50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) and then treated with 0.2 U/ml calf intestine alkaline phosphatase (Boehringer Mannheim) at 37°C for 30 min prior to Western blotting. In Fig. 8, the washed beads were suspended in 20 μl 2× SDS sample buffer, and the eluted proteins were analysed by gel electrophoresis and autoradiography.

Kinase Assays

Cdc2 and MAP kinase activities were measured with histone H1 and MBP (myelin basic protein), respectively. Lysate samples were first diluted 10-fold in kinase buffer (40 mM Hepes, pH 7.2, 20 mM $MgCl_2$, 10 mM EGTA) and then 5 μl was mixed with 5 μl of kinase buffer containing 0.2 mg/ml calf thymus histone H1 (Boehringer), 10 μM protein kinase A inhibitor (Sigma), and 0.5 mCi/ml [α-^{32}P]ATP (Amersham). The reactions were incubated at room temperature for 15 min and terminated by the addition of an equal volume of 2× SDS sample buffer. After electrophoresis in 12% polyacrylamide gels, the gels were dried and processed for autoradiography. MAP kinase assays were performed essentially as the H1 kinase assays except that MBP (Sigma) and $p27^{Kip1}$ (to inhibit cdc2 kinase activity) were present at 2.0 mg/ml and 10 μg/ml, respectively.

The MEK inhibitor PD98059 (2′-amino-3′-methoxyflavone; Calbiochem) was solubilized in DMSO and stored at −20°C. In control incubations, DMSO was added at a final concentration of 1% (v/v).

Preparation of His_6-$p27^{Kip1}$

His_6-$p27^{Kip1}$ in pET21d was transformed into *Escherichia coli* BL21(DE3)LysS and a 400-ml culture was grown at 37°C to an A_{600} of 0.8. Expression of the recombinant protein was induced with 100 μM IPTG at 25°C for 12–16 h. The cells were harvested and lysed with buffer I (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, and 5 mM imidazole) supplemented with 1 mM PMSF, 10 μg/ml leupeptin, and 1 mg/ml lysozyme at 4°C for 15 min. Following cell lysis with a french press, insoluble material was pelleted at 12,000*g* for 30 min and the supernatant was incubated at 80°C for 10 min as $p27^{Kip1}$ is a heat-stable protein (Polyak *et al.*, 1994). The heat-denatured proteins were pelleted at 12,000*g* for 30 min, and the supernatant was applied onto Ni^{2+}–NTA agarose (Qiagen) equilibrated with buffer I. The matrix was washed with 5 column volumes of buffer I, followed by 10 column volumes of buffer II (20 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 25 mM imidazole, 0.5% Triton X-100, and 0.5% Tween 20) and 5 column volumes of buffer I. His_6-$p27^{Kip1}$ was eluted with 20 mM Tris–HCl, pH 8.0, 0.5 M NaCl, and 150 mM imidazole, dialysed against T buffer (pH 8.0), and stored at −80°C. Column chromatography was carried out at 4°C.

RESULTS

Clam p82/CPEB Is Phosphorylated in pH-Activated Oocyte Lysates

Clam p82/CPEB is phosphorylated within minutes of fertilisation, on multiple residues, and its apparent molecu-

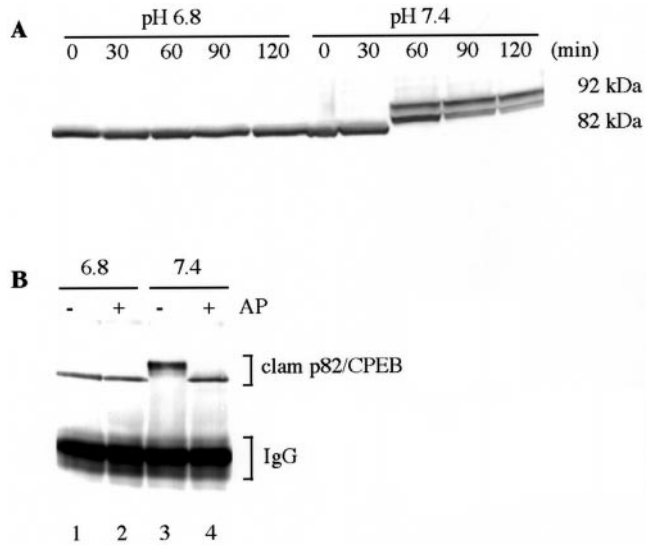

FIG. 1. Activation of clam p82/CPEB phosphorylation by elevated pH. (A) Oocyte cell-free extract was incubated at 18°C under control (pH 6.8) and activated (pH 7.4) conditions and samples taken at indicated times were analysed by Western blot using anti-clam p82/CPEB antibody. (B) Lysates under pH 6.8 (lanes 1 and 2) or pH 7.4 (lanes 1 and 3) conditions were incubated for 1 h and clam p82/CPEB was immunoprecipitated with anti-clam p82/CPEB antibody. The immunoprecipitates were used as the substrate for phosphatase treatment at 37°C for 30 min (lanes 2 and 4) and analysed by Western blotting.

lar weight increases from 82 to 92 kDa in SDS–PAGE (Walker *et al.*, 1999; data not shown). To examine the protein kinase(s) that phosphorylate clam p82/CPEB, we used an *in vitro* activation system from clam oocytes (Walker *et al.*, 1996) based on the natural permanent rise in pH that accompanies their maturation (reviewed by Baltz, 1993; Colas and Dubé, 1998). The intracellular pH of clam oocytes, pH 6.8–6.9, rises rapidly to pH 7.2 in the fertilised egg (Dubé, 1988; Dubé and Eckberg, 1997).

Cell-free extracts were made from clam oocytes in T pH 6.8 buffer (Materials and Methods). To mimic oocyte activation, an equal volume of either pH 6.8 (control) or pH 8.0 (activated) buffer was added to the concentrated extracts (final pH is 6.8 and 7.4, respectively), followed by incubation at 18°C for up to 2 h. Previously, using this *in vitro* activation system we showed that raising the pH activates the rate and pattern of protein synthesis, resulting from activation of kinases (Walker *et al.*, 1996). To examine clam p82/CPEB modification, aliquots were taken at indicated times for Western blot analysis using specific rabbit anti-p82 antibody. The mobility of clam p82/CPEB did not alter under control conditions (pH 6.8). However, under pH-activated conditions (pH 7.4) the apparent molecular weight of clam p82/CPEB increased from 82 to 92 kDa (Fig. 1A), mimicking the size change observed in activated oocytes (Walker *et al.*, 1996, 1999). Frequently, intermediate bands of clam p82/CPEB (85- to 87-kDa proteins) were detected as shown in Fig. 1A. Their presence and levels with respect to unmodified/fully modified proteins were variable; this variability reflected usage of different batches of oocyte extracts and/or gel systems (data not shown). To examine the nature of the upper bands of clam p82/CPEB, immunoprecipitates of lysates obtained after 1 h incubation with anti-p82 antibody and protein A–Sepharose were treated with calf intestine alkaline phosphatase. This treatment, which abolished the upper M_r bands and concentrated all detectable antigen into a sharp single band with the mobility of the control oocyte 82-kDa polypeptide (Fig. 1B), demonstrated unambiguously that the upper bands were due to phosphorylation. These data confirm our previous observations of clam p82 phosphorylation *in vitro*, detected indirectlythrough labelling of protein with labelled RNA and UV irradiation (Walker *et al.*, 1996).

Calcium Accelerates Clam p82/CPEB Phosphorylation in Vitro

The ionic events linked to activation of clam oocytes include a transient increased Ca^{2+} influx from external seawater in addition to an acid release. An activated Na^+/H^+ exchanger is responsible for the pH increase; however, the rise in pH is not sufficient for triggering GVBD, though it does allow its progression at an optimal rate (Dubé, 1988; Dubé and Eckberg, 1997). The pioneering work of Winkler and colleagues demonstrated that an increase in both intracellular pH and Ca^{2+} is required for full activation of the protein synthetic machinery and subsequent production of key proteins regulating ongoing mitotic cell cycles in fertilised sea urchins eggs (Winkler *et al.*, 1980; Winkler and Steinhardt, 1981).

The oocyte lysates used in Fig. 1 and previously (Walker *et al.*, 1996) were prepared in buffers lacking $CaCl_2$ and EGTA. To examine the effect of Ca^{2+} on clam p82/CPEB phosphorylation *in vitro*, we added up to 1 mM $CaCl_2$ to an extract that was then pH activated and analysed aliquots withdrawn at various times postactivation by Western blotting. Intriguingly, the timing of clam p82/CPEB phosphorylation was slightly accelerated when $CaCl_2$ was added to extracts, at all concentrations tested (0.1, 0.5, and 1.0 mM; Fig. 2A). This acceleration is seen particularly clearly at the 30-min time point, which shows a distinct p82 polypeptide of lower mobility (Fig. 2A). Clam p82/CPEB was not modified under control conditions (pH 6.8), even in the presence of 0.5 mM $CaCl_2$ (Fig. 2B). Note that the timing of phosphorylation differs slightly between the different batches of lysate used in these experiments (Figs. 2A and 2B).

Previously, we showed that cdc2 kinase is implicated in clam p82/CPEB phosphorylation *in vitro* and *in vivo* (Walker *et al.*, 1996, 1999). To examine whether the Ca^{2+}-activated protein kinase is cdc2 kinase or another kinase,

we used a recombinant His-tagged $p27^{Kip1}$ protein, a potent inhibitor of cdc2, cdk2, and cdk4 kinase (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994). We purified recombinant His_6-$p27^{Kip1}$ using heat treatment and Ni–NTA resin-affinity chromatography (see Materials and Methods). SDS–polyacrylamide gel electrophoresis analysis of column fractions showed that the 28-kDa $p27^{Kip1}$ protein, eluted with 150 mM imidazole-containing buffer, was extensively purified (Fig. 3A). The ability of $p27^{Kip1}$ to block cdc2 kinase activity was tested by its addition at various concentrations to a pH-activated oocyte lysate. After 1 h, samples were taken and H1 kinase activity was measured. While addition of up to 2.5 μg/ml (final concentration) of inhibitor did not significantly reduce cdc2 activity, complete kinase inhibition was achieved with 5 to 20 μg/ml of $p27^{Kip1}$ (Fig. 3B, a). Clam p82/CPEB phosphorylation correlated directly with the cdc2 kinase activity (Fig. 3B, a and b). Last, Ca^{2+} did not overcome $p27^{Kip1}$ inhibition of p82 phosphorylation (Fig. 3C, c and d). These data suggest that cdc2 kinase activity is required for phosphorylation of clam p82/CPEB and, second, that Ca^{2+} may accelerate the activation of cdc2 kinase *in vitro*.

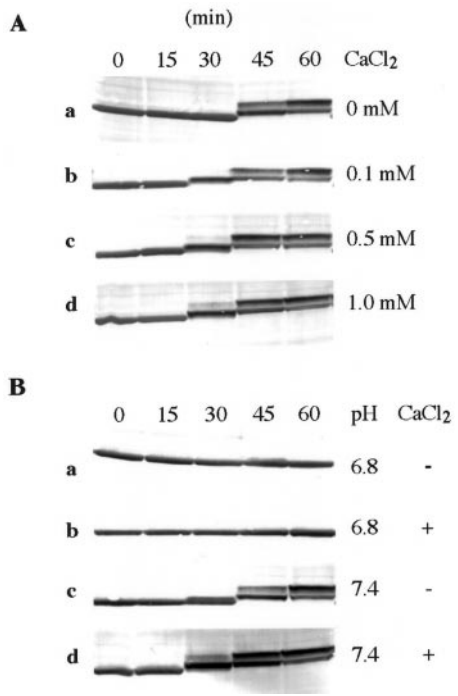

FIG. 2. Calcium hastens the phosphorylation of clam p82/CPEB. (A) Oocyte lysate was activated in pH 7.4 buffer containing various concentrations of added $CaCl_2$. a, no $CaCl_2$ independently; b, 0.1 mM; c, 0.5 mM; d, 1.0 mM (final concentration). Samples were taken for Western blot analysis with anti-clam p82/CPEB antibody at indicated times. (B) Oocyte lysates were incubated in pH 6.8 or pH 7.4 buffer containing $CaCl_2$ (0.5 mM). a, pH 6.8; b, pH 6.8 plus $CaCl_2$; c, pH 7.4; d, pH 7.4 plus $CaCl_2$.

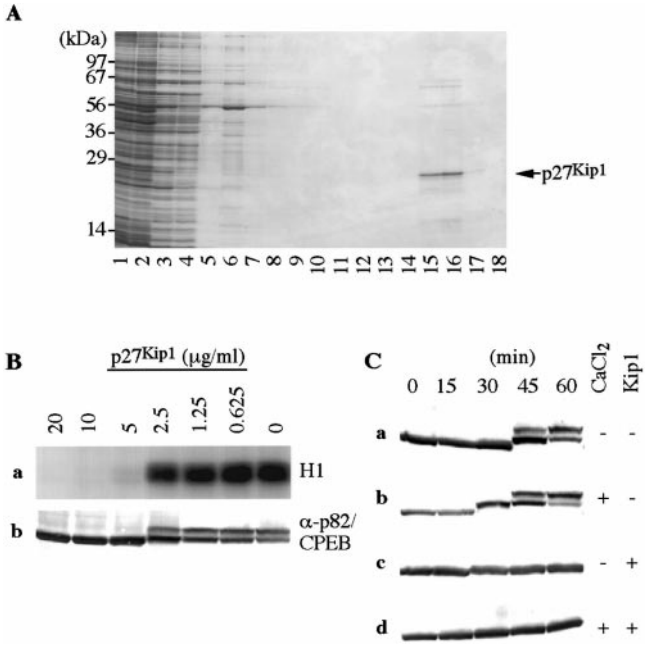

FIG. 3. $p27^{Kip1}$ inhibits both cdc2 kinase activity and clam p82/CPEB phosphorylation. (A) Purification of recombinant His_6-$p27^{Kip1}$. Whole-cell extract after induction with IPTG (lane 1), supernatant (lane 2), heat-treated extract (lane 3), flowthrough fraction from Ni-resin column (lane 4), wash fractions (lanes 5–14), and fraction eluted with 150 mM imidazole (lanes 15–18). (B) Oocyte extract was activated by incubation in pH 7.4 buffer containing various concentrations of $p27^{Kip1}$. After 1 h, histone H1 kinase activity (cdc2 kinase activity) was measured (a) or samples were analysed by immunoblot using anti-clam p82/CPEB antibody (α-p82/CPEB, b). (C) Oocyte lysate was activated in pH 7.4 buffer containing $CaCl_2$ (0.5 mM) and/or $p27^{Kip1}$ (10 μg/ml). a, pH 7.4; b, pH 7.4 plus $CaCl_2$; c, pH 7.4 plus $p27^{Kip1}$; d, pH 7.4 plus $CaCl_2$ and $p27^{Kip1}$.

Endogenous Calcium Is Necessary for Clam p82/CPEB Phosphorylation

We then examined whether endogenous calcium is necessary for clam p82/CPEB phosphorylation. To do so,EGTA, a specific chelator of calcium, was added (final 5 mM) to activated oocyte extracts. As seen previously, the timing of clam p82/CPEB phosphorylation was accelerated by the addition of $CaCl_2$ (Fig. 4A, a and b). However, when endogenous calcium was chelated with EGTA, clam CPEB phosphorylation did not occur, implying that Ca^{2+} not only accelerates, but was required for, p82 phosphorylation (Fig. 4A, c). Next, we determined the minimum inhibitory EGTA concentration; such a determination would in turn suggest the required level of Ca^{2+} for phosphorylation. EGTA (0–3.2 mM) was added to pH-activated oocyte lysates, in the presence or absence of 0.5 mM $CaCl_2$. As shown in Fig. 4B, 0.1 mM EGTA completely inhibits p82 phosphorylation in the absence

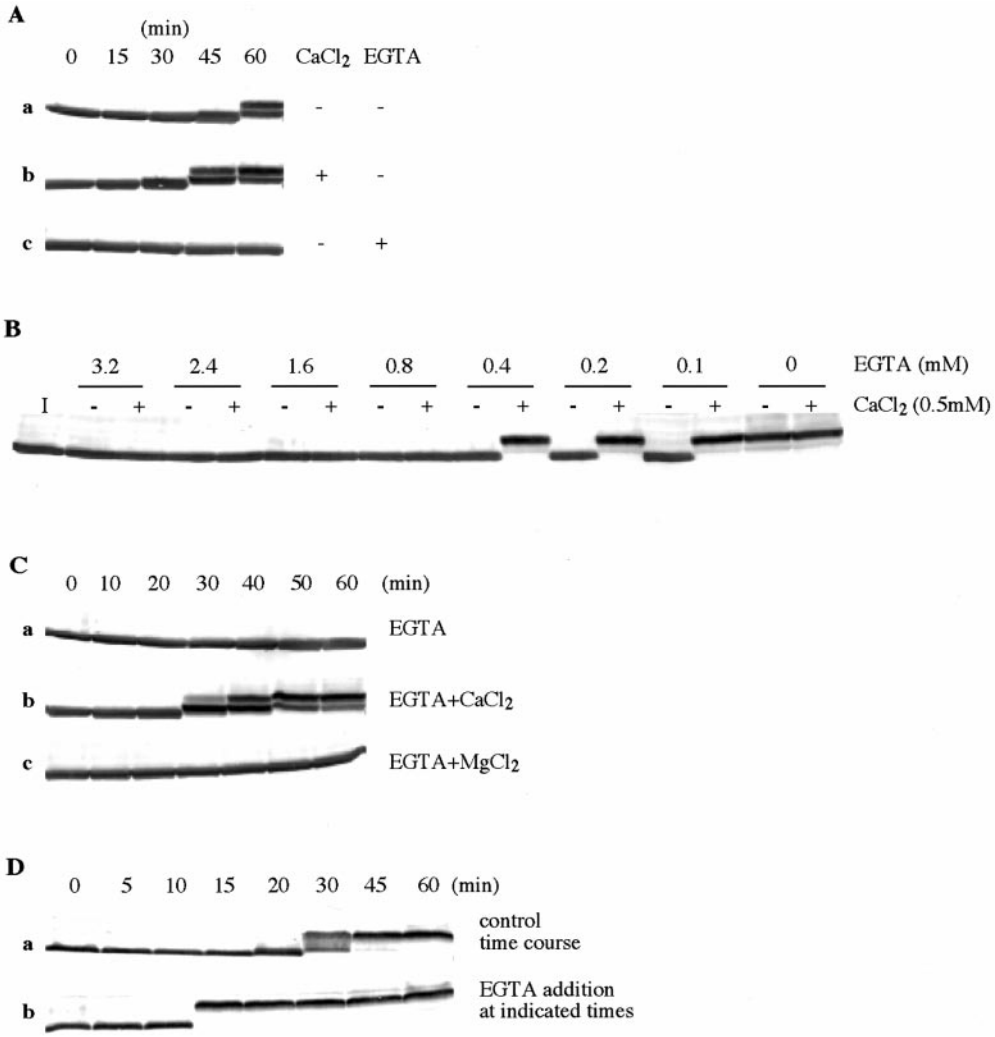

FIG. 4. Endogenous calcium is necessary for clam p82/CPEB phosphorylation. (A) Oocyte cell-free extract was activated in pH 7.4 buffer with or without $CaCl_2$ (0.5 mM) and EGTA (5 mM), and samples taken at indicated times were analysed by immunoblot using anti-clam p82/CPEB antibody. a, pH 7.4; b, pH 7.4 plus $CaCl_2$; c, pH 7.4 plus EGTA. (B) Oocyte extract was activated in pH 7.4 buffer with various concentrations of EGTA. $CaCl_2$ was added to all indicated samples (+) at a final concentration of 0.5 mM. After 1 h, samples were taken and analysed by immunoblotting using anti-clam p82/CPEB antibody. "I" indicates initial lysate. (C) Time course of clam p82/CPEB phosphorylation under activated conditions. a, plus EGTA; b, plus EGTA and $CaCl_2$; c, plus EGTA and $MgCl_2$ (EGTA, 0.4 mM; $CaCl_2$, 0.5 mM; $MgCl_2$, 0.5 mM). (D) EGTA-sensitive period of clam p82/CPEB phosphorylation. a, normal time course of clam p82/CPEB phosphorylation under activated conditions; b, EGTA (0.4 mM) was added to activated oocyte extract at indicated times; after 1 h, all samples were treated with SDS sample buffer and analysed by immunoblot using anti-clam p82/CPEB antibody.

of added calcium. This suggests that less than 0.1 mM Ca^{2+} is present in the oocyte extract and that this is indispensable for clam p82/CPEB phosphorylation under activated conditions. This estimate was confirmed in reactions containing both the cation and the chelator; p82 is phosphorylated as long as about 0.1 mM Ca^{2+} was in excess (Fig. 4B). We did not determine the absolute minimal levels of Ca^{2+} required. In investigating the cation specificity, we observed that while inhibition of clam p82/CPEB phosphorylation by EGTA was overcome by $CaCl_2$ addition (0.1 mM in excess over EGTA), when $MgCl_2$ (also 0.1 mM in excess over EGTA) was added to extracts containing EGTA, clam p82/CPEB phosphorylation did not occur (Fig. 4C). This confirms that the Ca^{2+} ion is necessary for clam p82/CPEB phosphorylation.

To determine the time window of sensitivity towards EGTA, oocyte extracts were activated under high pH conditions without additional calcium, and then 0.4 mM EGTA was added at each indicated time during activation. After 1 h, all samples were analysed by immunoblotting

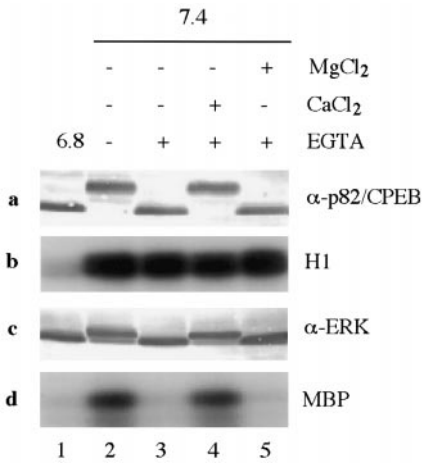

FIG. 5. Clam p82/CPEB phosphorylation directly correlates with MAP kinase activity. Oocyte extracts were incubated under control (pH 6.8) and activation (pH 7.4) conditions. After 1 h, samples were treated with SDS sample buffer for immunoblot analysis (a, α-p82/CPEB; c, α-ERK) or assayed for histone H1 kinase activity (b) and MBP kinase activity (MAP kinase activity, d). Lane 1, pH 6.8; lane 2, pH 7.4; lane 3, pH 7.4 plus EGTA; lane 4, pH 7.4 plus EGTA and $CaCl_2$; lane 5, pH 7.4 plus EGTA and $MgCl_2$ (EGTA, 0.4 mM; $CaCl_2$, 0.5 mM; $MgCl_2$, 0.5 mM).

using anti-p82 antibody. In the control experiment, activated extracts (no $CaCl_2$ and EGTA) were assayed at indicated times. Strikingly, phosphorylation of clam p82/CPEB was completely blocked by EGTA addition at 0, 5, and 10 min after activation but was quite unaffected by addition at 15 min or subsequently (Fig. 4D, b). We concluded that the calcium-dependent process for clam p82/CPEB phosphorylation (or activation of clam p82/CPEB kinase) is an early step *in vitro.*

Calcium Is Necessary for MAP Kinase Activation, But Not Cdc2 Kinase Activation in Vitro

To investigate the effect of EGTA treatment upon the activation of cdc2 kinase, histone H1 kinase activity was measured in oocyte extracts 1 h after activation. H1 kinase activity is elevated under high-pH conditions compared to control, lower pH conditions (Fig. 5, b, and Walker *et al.*, 1996). Contrary to our expectations that EGTA (without additional Ca^{2+}) may prevent the activation of cdc2 kinase, there were no remarkable changes in histone H1 kinase activity under high-pH conditions, irrespective of the presence of EGTA/$CaCl_2$/$MgCl_2$ (Fig. 5, b). Indeed, in this experiment cdc2 kinase activity does not directly correlate with clam p82/CPEB phosphorylation (compare a and b in Fig. 5). This result implies that a protein kinase(s) other than cdc2 kinase is also necessary for clam p82/CPEB phosphorylation and that the activation of this protein kinase depends on intracellular calcium. A number of different kinases are known to be activated during clam oocyte maturation, including protein kinase C, cdc2 kinase, and MAP kinase (Eckberg *et al.*, 1987; Draetta *et al.*, 1989; Shibuya *et al.*, 1992). The activation of a 42-kDa mitogen-activated protein kinase is a very early event following fertilisation of clam oocytes (Shibuya *et al.*, 1992) and in activated oocyte lysates (Walker *et al.*, 1996). Both tyrosine phosphorylation of clam MAPK and concomitant kinase activation begin within 2–3 min of fertilisation, peak at 15 min, then rapidly decline and disappear at the end of meiosis I (Shibuya *et al.*, 1992). MAP kinase activation appears to precede that of cdc2 kinase by several minutes. Maximal cdc2 activity is observed at GVBD, around 8–10 min postfertilisation (Turner *et al.*, 1995; Walker *et al.*, 1999). Our particular interest in MAP kinase was stimulated by reports that increased intracellular Ca^{2+} can modulate this kinase pathway in diverse cell types, including human fibroblasts and *Xenopus* oocytes (Chao *et al.*, 1992; Enslen *et al.*, 1996; Duesberry and Masui, 1996).

Two independent approaches were employed to assay MAP kinase activity in clam lysates. The first takes advantage of a size shift in MAP kinase on activation, due to tyrosine phosphorylation, and employs Western blot analysis using anti-ERK antibodies (Shibuya *et al.*, 1992; Walker *et al.*, 1996). The second method involves a direct kinase assay using MBP as the substrate for MAP kinase. As cdc2 kinase can also phosphorylate MBP (Shibuya *et al.*, 1992; data not shown), we used recombinant $p27^{Kip1}$ to block cdc2 kinase in MBP assays (see Fig. 3). Both approaches indicate the following. First, MAP kinase is activated under elevated pH conditions. Second, in the EGTA-treated lysates, MAP kinase is completely inhibited unless $CaCl_2$, but not $MgCl_2$, is also present (Fig. 5, c and d). Thus, MAP kinase activity, rather than cdc2 kinase, directly correlates with clam p82/CPEB phosphorylation in these assays.

To confirm that MAP kinase participates in clam p82/CPEB phosphorylation, we used the inhibitor PD98059 to selectively block the activity of MEK, the kinase that directly activates ERK-like MAP kinase (Alessi *et al.*, 1995; Dudley *et al.*, 1995). The lowest PD98059 concentration that completely inhibits MAP kinase activation was determined to be 50 μM in starfish oocytes (Sadler and Ruderman, 1998) and 30 μM in oocytes of *Chaetopterus* (Eckberg, 1997). PD98059 did inhibit the activation of clam MAP kinase in clam lysates (Fig. 6). However, complete inhibition of MAP kinase in clam oocyte extracts was not achieved, even at the highest concentration of PD98059 tested (100 μM; Fig. 6A, a and b). We do not understand why PD98059 does not effectively inhibit clam MAP kinase, even with preincubation or at higher concentrations of inhibitor (data not shown). We note that the major difference between our work and that of Eckberg (1997) and Sadler and Ruderman (1998), other than the organisms, was the use of cell-free lysates rather than cells. Whether this has a bearing on the issue is not known. Though phosphorylation of clam p82/CPEB did occur in the presence of

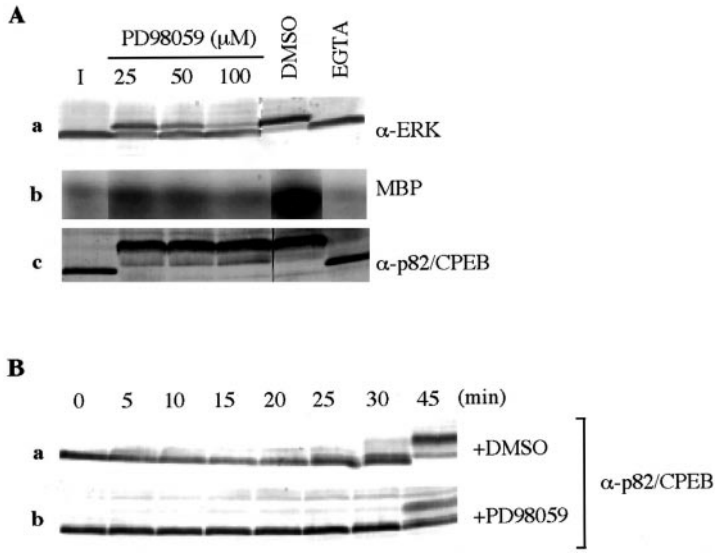

FIG. 6. MAPKK inhibitor partially inhibits MAP kinase activation and delays the timing of clam p82/CPEB phosphorylation. (A) MAPKK inhibitor PD98059 partially inhibits MAP kinase activity. Various concentrations of PD98059 in DMSO were added to oocyte extract incubated in pH 7.4 buffer as shown. After 1 h, samples were treated with SDS sample buffer for immunoblot using α-ERK (a) and α-p82/CPEB (c) antibodies or assayed for MAP kinase activity (b). "I" indicates initial lysate. (B) Time course of clam p82/CPEB phosphorylation in pH 7.4 buffer without (a) or with (b) MAPKK inhibitor PD98095 (100 μM). Samples were taken at indicated times and analysed by immunoblotting using α-p82/CPEB antibody.

inhibitor, this was apparently partial since in these lysates, the intermediate band of clam p82/CPEB increased in proportion to the concentration of inhibitor (Fig. 6A, c; also see Fig. 6B). PD98059 did not inhibit clam H1 kinase activity (data not shown).

The effect of the inhibitor on clam p82/CPEB phosphorylation was assayed in oocyte extracts incubated with or without 100 μM PD98059 for various times. The mobility shift of clam p82/CPEB which begins at 25 min after activation in the control-activated lysate is essentially complete by 45 min. In the inhibitor-treated extract, however, the unmodified form of clam p82/CPEB still remains at 45 min after activation (Fig. 6B). Thus, PD98059 delays the timing of clam p82/CPEB phosphorylation, suggesting that MAP kinase is a strong candidate for a clam p82/CPEB kinase.

MAP Kinase Activation and Inactivation in the Clam Oocyte Cell-Free System

As discussed earlier, the activation of clam MAP kinase at fertilisation appears to be a transient, one-time early event linked to release from cell cycle arrest (Shibuya *et al.*, 1992). To examine the kinetics of MAP kinase activation *in vitro*, oocyte extracts were incubated at pH 7.4 and samples were taken at intervals for Western blot analysis using anti-p82 and anti-ERK antibodies and for MBP kinase assays (Fig. 7A). In this experiment, clam p82/CPEB begins to be phosphorylated at 20 min (Fig. 7A, a). MAP kinase activation, noticeable within 5–10 min of incubation, clearly precedes p82 phosphorylation *in vitro*. Interestingly, MAP kinase activity is transient, with maximum activity detected around 40 min after activation, followed by a gradual decline to preactivation levels by around 100 min. Evidently, though the time course of MAP kinase activation *in vitro* is more prolonged than *in vivo* (Shibuya *et al.*, 1992), the cell-free system faithfully mimics the transient state of activation and suggests that the same regulatory processes are at work in both cases. Comparison of b and c in Fig. 7A implies that the decline in kinase activity is due to or accompanied by tyrosine/threonine dephosphorylation. We also found that MAP kinase activation in clam lysates is independent of protein synthesis (data not shown), just as it is *in vivo* (Ruderman *et al.*, 1991; Shibuya *et al.*, 1992).

Neither MAP kinase activation nor clam p82/CPEB phosphorylation occurred in EGTA-treated lysates, as shown in Fig. 5. To examine the relationship between the calcium-dependent period for clam p82/CPEB phosphorylation and for MAP kinase activation, 0.4 mM EGTA was added to an activated extract at frequent intervals and after 1 h samples were collected and assayed for MAP kinase activity. In this experiment, interestingly, we found that MAP kinase activity is low in extracts to which EGTA was added, up to 45 min (data not shown; see Fig. 8B). To confirm this result, we added EGTA to an activated oocyte extract at 40 min after incubation. Samples were taken at indicated times and

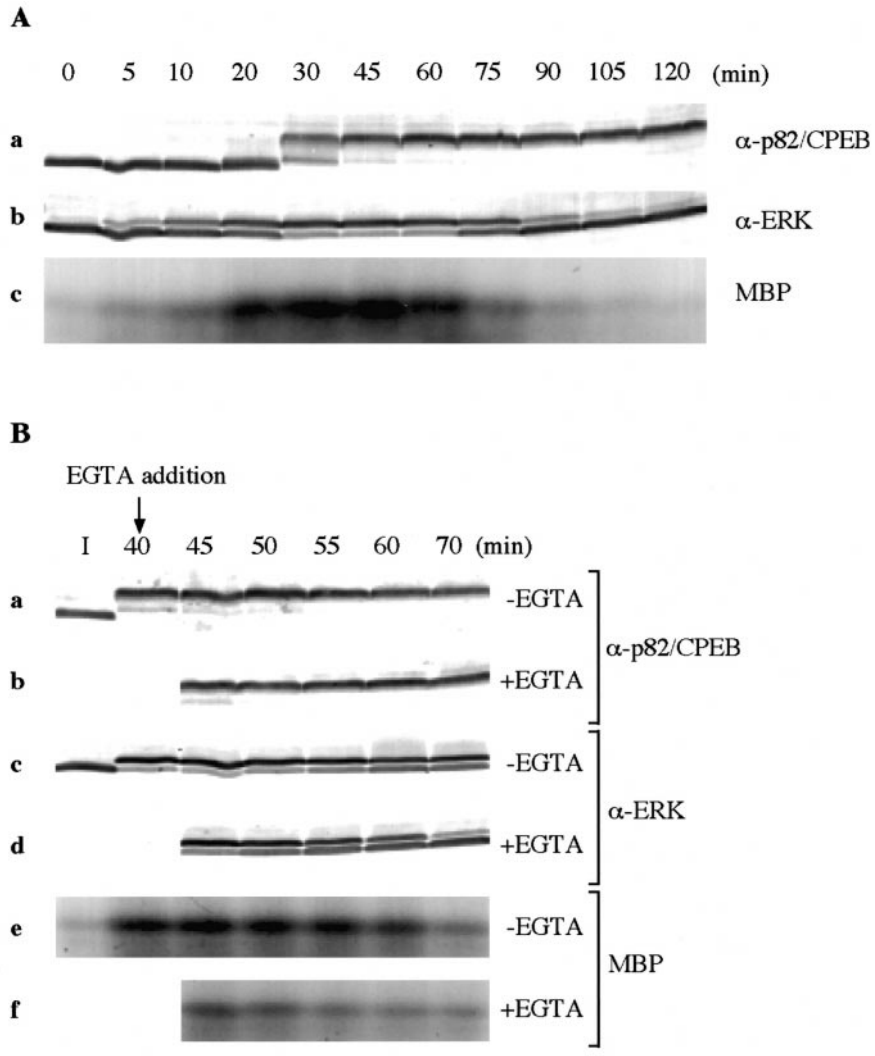

FIG. 7. MAP kinase activation in clam oocyte cell-free system. (A) Time course of MAP kinase activation in pH-activated oocyte lysates. Samples were taken at indicated times and analysed by immunoblotting using α-p82/CPEB (a) and α-ERK (b) antibodies or by MAP kinase assays (c). (B) Calcium depletion induces MAP kinase inactivation. EGTA (0.4 mM) was added to oocyte extracts after 40 min of activation, and samples were taken at indicated times and analysed as in A (e and f). "I" indicates initial lysate.

analysed by Western blotting (Fig. 7B, a–d) and in a kinase assay (Fig. 7B, e and f). When added at 40 min, EGTA did not affect p82/CPEB phosphorylation (Fig. 7B, a and b). MAP kinase in a normal lysate is maximally active 50 min postactivation, and then its activity decreases gradually (Fig. 7B, c and e). In contrast, in EGTA-treated extracts, MAP kinase activity shows a rapid decrease (Fig. 7B, d and f). One simple interpretation of these results is that MAP kinase activates a MAP kinase phosphatase through a Ca^{2+}-independent pathway. While EGTA abolishes the MAP kinase activation cascade, MAP kinase phosphatase activity is unaffected and may inactivate MAP kinase *in vitro*.

Both Cdc2 Kinase and MAP Kinase Are Necessary for Hyperphosphorylation of Clam p82/CPEB in Vitro

Previously we showed that $p27^{Kip1}$, a cdk inhibitor, inhibits the phosphorylation of clam p82/CPEB, implying that cdc2 kinase is required for this modification (Fig. 3). Subsequently, we demonstrated that Ca^{2+}-mediated activation of MAP kinase is also necessary for clam p82/CPEB phosphorylation (Figs. 4–6). We thus set out to address the relationship between the two kinases and clam p82/CPEB phosphorylation. In this experiment, three kinds of pH-activated oocyte extract, control extracts (no addition), extracts with

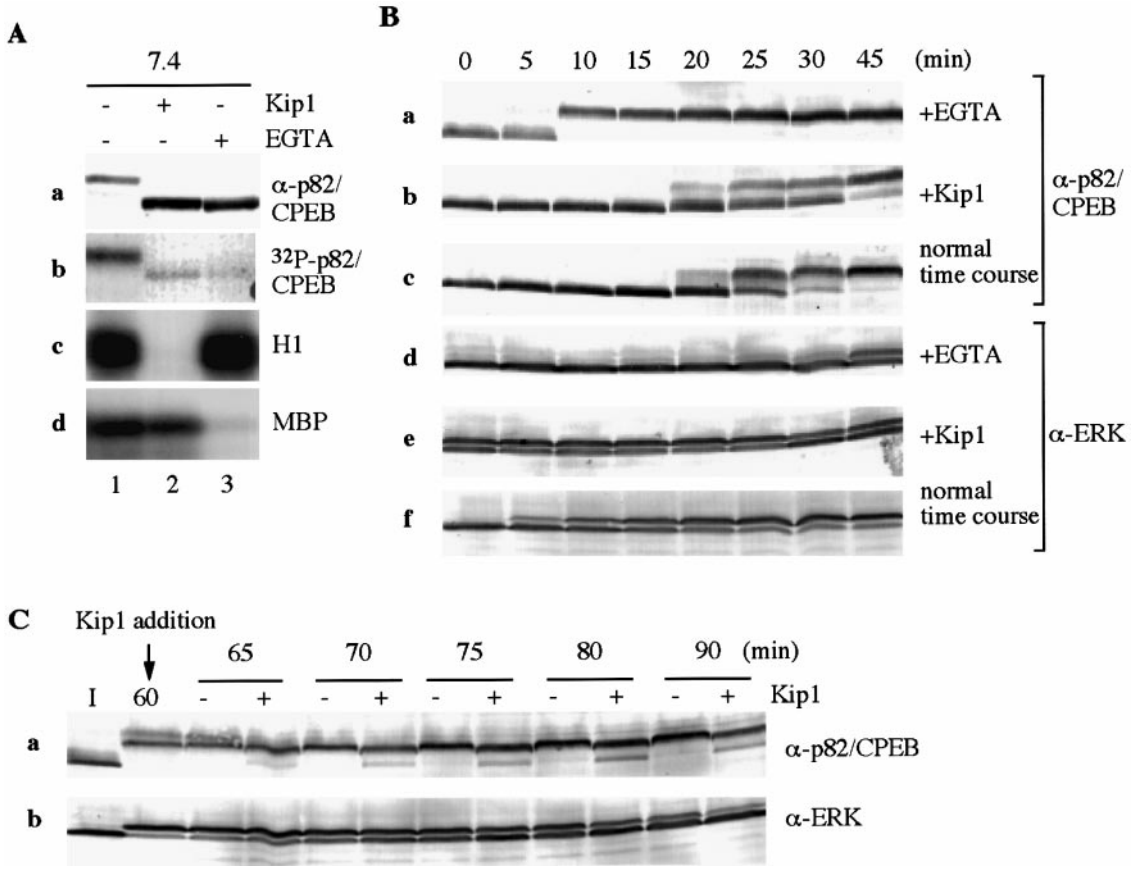

FIG. 8. Both cdc2 kinase and MAP kinase are necessary for hyperphosphorylation of clam p82/CPEB. (A) Oocyte extract was incubated at 18°C under activated conditions. $p27^{Kip1}$ or EGTA was added to extracts to inhibit cdc2 kinase (lane 2) or MAP kinase (lane 3), respectively. After 1 h, samples were taken for immunoblot analysis using α-CPEB (a) or for cdc2 kinase (c) and MAP kinase (d) assays. To examine the incorporation of ^{32}P into clam p82/CPEB, each sample was incubated in the presence of [α-^{32}P]ATP. After incubation, samples were immunoprecipitated with anti-clam p82/CPEB antibody, and precipitated materials were analysed by 10% SDS–PAGE and autoradiography (b). (B) EGTA- and $p27^{Kip1}$-sensitive periods of clam p82/CPEB phosphorylation. EGTA (0.4 mM, a and d) or $p27^{Kip1}$ (10 μg/ml, b and e) was added to activated oocyte extract at indicated times. After 1 h, samples were analysed by immunoblot using α-p82/CPEB (a and b) and α-ERK antibodies (d and e). For the control experiment, activated oocyte extracts were taken at indicated times and treated with SDS sample buffer for immunoblot analysis using α-p82/CPEB (c) and α-ERK (f). (C) Cdc2 kinase is necessary for the maintenance of hyperphosphorylation of clam p82/CPEB. $p27^{Kip1}$ was added to oocyte extracts 1 h after activation, and samples were taken at indicated times and analysed for immunoblotting using α-p82/CPEB (a) and α-ERK (b) antibodies. "I" indicates initial lysate.

added $p27^{Kip1}$, and extracts with added EGTA, were prepared for analysis of clam p82/CPEB phosphorylation. In the normal activated extract, both cdc2 kinase and MAP kinase are activated; clam p82/CPEB is phosphorylated and changes its molecular weight from 82 to 92 kDa (Fig. 8A, lane 1, a, c, and d). The $p27^{Kip1}$-treated extract has high MAP kinase activity, but not cdc2 kinase activity, while the EGTA-treated extract has high cdc2 kinase activity, but not MAP kinase activity (Fig. 8A, lanes 2 and 3, c and d). This result strongly suggests that the two kinases are activated by independent pathways. Clam p82/CPEB does not change in apparent molecular weight in either $p27^{Kip1}$- or EGTA-treated extracts (Fig. 8A, a). The incorporation of [α-^{32}P]ATP into the 92-kDa protein immunoprecipitated using anti-clam p82/CPEB antibody was detected (Fig. 8A, b, lane 1). Interestingly, we found that [α-^{32}P]ATP is incorporated into the 82-kDa form of clam CPEB in $p27^{Kip1}$- and to a lesser extent in EGTA-treated extracts, though not as efficiently as into the 92-kDa form (Fig. 8A, b, lanes 2 and 3), implying that clam p82/CPEB is in fact phosphorylated before any apparent size change.

Next, we determined the EGTA- and $p27^{Kip1}$-sensitive periods of clam p82/CPEB phosphorylation. Oocyte extracts were activated with high pH and EGTA or $p27^{Kip1}$ was added at indicated times. One hour after activation, the inhibitor-treated lysates were analysed by Western blotting using anti-clam p82/CPEB and anti-ERK antibodies and compared to control lysates (Fig. 8B). These data indicate that the

p27^{Kip1}-sensitive period is more extended than the EGTA-sensitive period and that the timing of clam p82/CPEB phosphorylation in the p27^{Kip1}-containing lysate corresponds with the control time course (Fig. 8B, a, b, and c). Furthermore, confirming the result shown in Fig. 8A, p27^{Kip1} does not prevent MAP kinase activation (Fig. 8B, e).

When p27^{Kip1} was added to oocyte extracts following a 60-min postactivation incubation, a low-molecular-weight form (intermediate form) of clam p82/CPEB appeared soon after (Fig. 8C, a). This result suggests that a clam p82/CPEB phosphatase is present in oocyte extracts and that the size shift of clam p82/CPEB from 82 to 92 kDa is due to phosphorylation by cdc2 kinase. Furthermore, the active form of MAP kinase decreases in p27^{Kip1}-treated extracts compared with control extracts (Fig. 8C, b). It is possible therefore that cdc2 kinase plays a role in the maintenance of MAP kinase activity, but not in MAP kinase activation.

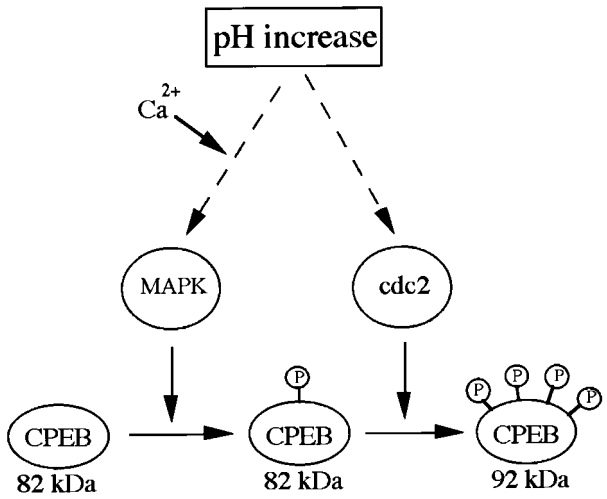

FIG. 9. A model of clam p82/CPEB phosphorylation by MAP kinase and cdc2 kinases mediated by elevated Ca^{2+} and pH following oocyte activation *in vitro.*

DISCUSSION

In this paper, we focused on the phosphorylation of clam p82/CPEB, an important regulator of gene expression in early development. In maturing clam oocytes, p82/CPEB shifts in apparent size by 10 kDa within minutes of fertilisation, in steps, consistent with being phosphorylated on multiple residues. Our interest in identifying the kinases(s) and the role of phosphorylation in p82/CPEB function is based on the following. Phosphorylation of p82/CPEB precedes and, according to inhibitor studies *in vitro* and *in vivo,* may be required for translational activation of masked maternal mRNAs. Indeed, phosphorylation bridges the two functions of p82/CPEB, as a translational repressor in the oocyte and as a polyadenylation factor in the activated egg, and may mediate changing protein–protein interactions, such as with CPSF subunits and/or poly(A) polymerase. Last, fully modified p92/CPEB is degraded during the first mitotic cell division, at about the time that cyclins are destroyed (Walker *et al.*, 1996, 1999; Minshall *et al.*, 1999).

Previously, we developed an *in vitro* activation cell-free system from clam oocytes, based on the natural rise in pH that accompanies *Spisula* oocyte activation (Walker *et al.*, 1996). In extending this work here, we demonstrate that endogenous Ca^{2+} (100 μM or less) in addition to raised pH is required for p82/CPEB phosphorylation and show that both cdc2 kinase and MAP kinases, acting independently, are necessary for its hyperphosphorylation. These conclusions are based on the following observations. EGTA prevents clam p82/CPEB hyperphosphorylation, whereas histone H1 kinase activity (cdc2 kinase activity) is not affected by EGTA; absence of Ca^{2+} prevents MAP kinase activation (Figs. 4 and 5). p27^{Kip1}, the specific cdk inhibitor, blocks both cdc2 kinase activity and the hyperphosphorylation of clam p82/CPEB, though MAP kinase activation under these conditions proceeds as usual (Figs. 3 and 5). Finally, ^{32}P incorporation into clam p82/CPEB occurs in both p27^{Kip1}- and EGTA-treated oocyte extracts, though the labelled protein does not alter its migration. Moreover, the p27^{Kip1}-sensitive period of clam p82/CPEB hyperphosphorylation is significantly prolonged compared with the EGTA-sensitive period (Fig. 8). These results lead us to propose the following model (Fig. 9). Subsequent to a pH increase and in the presence of Ca^{2+}, MAP and cdc2 kinases are independently activated in *Spisula.* Initially, MAP kinase recognises and phosphorylates clam p82/CPEB at one (few?) site that does not alter its mobility in polyacrylamide gels. Cdc2 kinase is then able to modify this minimally phosphorylated form of clam p82/CPEB to result in the hyperphosphorylated form with its distinctive 10-kDa apparent size jump in denaturing polyacrylamide gels. Our data regarding the relative timing of kinase activation are entirely consistent with *in vivo* observations (Shibuya *et al.*, 1992; Walker *et al.*, 1999).

The role of cdc2 kinase in clam p82/CPEB phosphorylation, documented here using a specific cdk inhibitor, extends previous inhibitor studies *in vitro* and *in vivo.* The timing of clam p82/CPEB phosphorylation, initiated at GVBD, also correlates with cdc2 kinase activation (Walker *et al.*, 1996, 1999). Moreover, *Xenopus* CPEB, a 62-kDa protein, is phosphorylated to a 64/65-kDa species at GVBD, approximately 4–5 h after progesterone treatment (Hake and Richter, 1994; de Moor and Richter, 1997). The oocyte protein can be induced to have a slower mobility when extracts are supplemented with baculovirus-expressed cyclin or p34^{cdc2} (Paris *et al.*, 1991). Both the timing and its ability to act as a substrate suggest that *Xenopus* CPEB is phosphorylated by cdc2.

Our identification of clam MAP kinase as the EGTA-sensitive kinase is based on the direct correlation between MBP kinase activity and p82/CPEB phosphorylation (Figs. 5 and 8), the significant (though incomplete) inhibition of p82 phosphorylation by PD98059, a specific MAP kinase inhib-

itor (Fig. 6), and the relative extent of EGTA- and $p27^{Kip1}$-sensitive periods of p82 phosphorylation, compared with the known timing of activation of MAP and cdc2 kinases *in vivo* (Fig. 8). Nevertheless, we cannot exclude the possibility that other Ca^{2+}-dependent kinases also play a part in p82/CPEB phosphorylation. For example, one common mechanism by which elevated Ca^{2+} regulates cellular events is through its interaction with calmodulin. The binary complex modulates the activity of several regulatory proteins, including calcium/calmodulin-dependent protein kinase (Enslen *et al.*, 1996; Muthalif *et al.*, 1996). Clearly, the identification of p82/CPEB phosphorylation sites coupled with the direct examination of soluble recombinant clam p82 (not yet available) modification by MAP and cdc2 kinases will ultimately settle this question, one way or the other. Preliminary data indicate that mutant constructs of p82 lacking nine proline-directed Ser/Thr phosphorylation (S/TP) sites, characteristic of cdc2 and MAP kinase sites (though not of CaMKII), are not modified in progesterone-treated maturing *Xenopus* oocytes, unlike the wild-type protein and the endogenous *Xenopus* CPEB (George Thom and N.S., unpublished results).

Another important question addresses the role of phosphorylation in CPEB function. An attractive model postulates that phosphorylation enhances the protein's ability to act as a specificity factor in cytoplasmic polyadenylation (Paris *et al.*, 1991), for example, by altering its affinity towards CPSF (cleavage and polyadenylation specificity factor) subunits and/or poly(A) polymerase (Bilger *et al.*, 1994). This possibility has been ruled out in recent experiments, at least in the case of *Xenopus* class I mRNAs, such as those encoding *c-mos* and cyclin A1, consistent with the observation that their polyadenylation precedes CPEB phosphorylation and is in fact independent of cdc2 (de Moor and Richter, 1997; Ballantyne *et al.*, 1997).

CPEB modification may alter its RNA binding, though the existing evidence is not clear-cut. On the one hand, p82 phosphorylation alters RNP complex formation in gel retardation gels between the lysate protein and the 3′ UTR masking element RNA probe, suggestive of reduced protein–RNA and/or protein–protein contacts (Walker *et al.*, 1996). In polysome gradients, the phosphorylated protein is not associated with translationally activated mRNA in clam egg lysates (Minshall *et al.*, 1999), implying that modification may reduce its RNA binding. However, we (Walker *et al.*, 1996) and Hake *et al.* (1998) have also noted that the clam and frog proteins are able to UV cross link to their target RNAs essentially with similar efficiency regardless of whether the protein is modified. Direct examination of the RNA-binding properties of the two forms of recombinant protein (from baculovirus-virus infected Sf9 insect cells, N.M., unpublished) may help to resolve the issue.

Finally, phosphorylation may target p82/CPEB as a substrate of the ubiquitin-mediated proteasome pathway. This pathway is implicated in p82 degradation so far only indirectly, based partly on the similar timing of proteolysis of p82 and cyclins in clam embryos and partly on the conservation of a PEST-type motif in CPEB family members (Walker *et al.*, 1999). Preliminary observations, showing the stabilisation of clam p82/CPEB in maturing *Xenopus* oocytes by mutagenesis of S/TP phosphorylation sites, lend further support for this proposed role (George Thom and N.S., unpublished results). Moreover, recent studies demonstrating the role of MAP and cdc2 kinases in BCL-6 transcription factor and Sic1 degradation by the ubiquitin-proteasome pathway (Niu *et al.*, 1998; Nishizawa *et al.*, 1998) underscore the importance of this kinase combination in mediating proteolysis.

ACKNOWLEDGMENTS

We thank Dr. J. V. Ruderman for helpful suggestions and encouragement at the MBL in Woods Hole, Massachusetts; Dr. M. Carrington for insightful discussions; and Drs. M. Howell and T. Hunt for generously providing the His_6-$p27^{Kip1}$ expression vector. Dr. M. Yamashita provided much useful advice on protein kinases. We are also grateful to members of the R. J. Jackson, C. W. J. Smith, and N. Standart laboratories for technical advice and encouragement. Y.K. is a Research Fellow of the Japan Society for the Promotion of Science. Work in Cambridge was funded by an MRC project grant (N.S.).

REFERENCES

Alessi, D. F., Cuneda, A., Cohen, P., Dudly, D. T., and Saltiel, A. R. (1995). PD 98059 is a specific inhibitor of the activation of mitogen-activated protein kinase *in vitro* and *in vivo*. *J. Biol. Chem*. **270,** 27489–27494.

Allen, R. D. (1953). Fertilisation and artificial activation in the egg of the surf clam, *Spisula solidissima*. *Biol. Bull.* **105,** 213–239.

Ballantyne, S., Daniel, D. L., and Wickens, M. (1997). A dependent pathway of cytoplasmic polyadenylation reactions linked to cell cycle control by c-*mos* and CDK1 activation. *Mol. Biol. Cell* **8,** 1633–1648.

Baltz, J. M. (1993). Intracellular pH regulation in the early embryo. *BioEssays* **15,** 523–530.

Bilger, A., Fox, C. A., Wahle, E., and Wickens, M. (1994). Nuclear polyadenylation factors recognize cytoplasmic polyadenylation element. *Genes Dev*. **8,** 1106–1116.

Chao, T.-S. O., Byron, K. L., Lee, K.-M., Villereal, M., and Rosner, M. R. (1992). Activation of MAP kinases by calcium-dependent and calcium-independent pathways. *J. Biol. Chem*. **260,** 13947–13954.

Colas, P., and Dubé, F. (1998). Meiotic maturation in mollusc oocytes. *Semin. Cell Dev. Biol*. **9,** 539–548.

Curtis, D., Lehmann, R., and Zamore, P. (1995). Translational regulation in development. *Cell* **81**, 171–178.

de Moor, C., and Richter, J. D. (1997). The mos pathway regulates cytoplasmic polyadenylation in *Xenopus* oocytes. *Mol. Cell. Biol*. **17,** 6419–6426.

Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J., and Beach, D. (1989). Cdc2 protein kinase is complexed with both cyclin A and B: Evidence for proteolytic inactivation of MPF. *Cell* **56,** 829–838.

Dubé, F. (1988). The relationships between early ionic events, the pattern of protein synthesis, and oocyte activation in the surf clam, *Spisula solidissima*. *Dev. Biol.* **126,** 233–241.

Dubé, F., and Eckberg, W. R. (1997). Intracellular pH increase driven by an Na^+/H^+ exchanger upon activation of surf clam oocytes. *Dev. Biol.* **190,** 41–54.

Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* **92,** 7686–7689.

Duesbery, N. S., and Masui, Y. (1996). The role of microtubules and inositol triphosphate induced Ca^{2+} release in the tyrosine phosphorylation of mitogen-activated protein kinase in extracts of *Xenopus laevis* oocytes. *Zygote* **4,** 21–30.

Eckberg, W. R. (1997). MAP and cdc2 kinase activities at germinal vesicle breakdown in *Chaetopterus*. *Dev. Biol.* **191,** 182–190.

Eckberg, W. R., Szuts, E. Z., and Carroll, A. G. (1987). Protein kinase C activity, protein phosphorylation, and germinal vesicle breakdown in *Spisula* oocytes. *Dev. Biol.* **127,** 57–64.

Enslen, H., Tokumitsu, H., Stork, P. J., Davis, R. J., and Soderling, T. R. (1996). Regulation of mitogen-activated protein kinases by a calcium/calmodulin-dependent protein kinase cascade. *Proc. Natl. Acad. Sci. USA* **93,** 10803–10808.

Gebauer, F., Xu, W., Cooper, G., and Richter, J. (1994). Translational control by cytoplasmic polyadenylation of c-*mos* mRNA is necessary for oocyte maturation in the mouse. *EMBO J.* **13,** 5712–5720.

Hake, L. E., and Richter, J. D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. *Cell* **79,** 617–627.

Hake, L. E., Mendez, R., and Richter, J. D. (1998). Specificity of RNA binding by CPEB: Requirement for RNA recognition motifs and a novel zinc finger. *Mol. Cell. Biol.* **18,** 685–693.

Harlow, E., and Lane, D. (1988). "Antibodies: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hunt, T., Luca, F. C., and Ruderman, J. V. (1992). The requirement for protein synthesis and degradation, and the control of destruction of cyclins A and B in the meiotic and mitotic cell cycles of the clam embryo. *J. Cell Biol.* **116,** 707–724.

Katsu, Y., Yamashita, M., Kajiura, H., and Nagahama, Y. (1993). Behavior of the components of maturation-promoting factor, cdc2 kinase and cyclin B, during oocyte maturation of goldfish. *Dev. Biol.* **160,** 99–107.

Kondo, T., Yanagawa, T., Yoshida, N., and Yamashita, M. (1997). Introduction of cyclin B induces activation of the maturation-promoting factor and breakdown of germinal vesicle in growing zebrafish oocytes unresponsive to the maturation-inducing hormone. *Dev. Biol.* **190,** 142–152.

Kyriakis, J. M., and Avruch, J. (1996). Protein kinase cascades activated by stress and inflammatory cytokines. *BioEssays* **18,** 567–577.

Minshall, N., Walker, J., Dale, M., and Standart, N. (1999). Dual roles of p82, the clam CPEB homologue, in cytoplasmic polyadenylation and translational masking. *RNA* **5,** 27–38.

Muthalif, M. M., Benter, I. F., Uddin, M. R., and Malik, K. U. (1996). Calcium/calmodulin-dependent protein kinase IIa mediates activation of mitogen-activated protein kinase and cytosolic phospholipase A_2 in norepinephrine-induced arachidonic acid release in rabbit aortic smooth muscle cells. *J. Biol. Chem.* **271,** 30149–30157.

Nagahama, Y., Yoshikuni, M., Yamashita, M., Tokumoto, T., and Katsu, Y. (1995). Regulation of oocyte maturation in fish. *Curr. Top. Dev. Biol.* **30,** 103–145.

Nishida, E., and Gotoh, Y. (1993). The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.* **18,** 128–131.

Nishizawa, M., Kawasumi, M., Fujino, M., and Toh-e, A. (1998). Phosphorylation of Sic1, a cyclin-dependent kinase (cdk) inhibitor, by cdk including Pho85 kinase is required for its prompt degradation. *Mol. Biol. Cell* **9,** 2393–2405.

Niu, H., Ye, B. H., and Dalla-Favera, R. (1998). Antigen receptor signaling induces MAP kinase-mediated phosphorylation of the BCL-6 transcription factor. *Genes Dev.* **12,** 1953–1961.

Paris, J., Swenson, K., Piwnica-Worms, H., and Richter, J. D. (1991). Maturation-specific polyadenylation: *In vitro* activation by $p34^{cdc2}$ and phosphorylation of a 58-kD CPE-binding protein. *Genes Dev.* **5,** 1697–1708.

Picard, A., Galas, S., Peaucellier, G., and Doree, M. (1996). Newly assembled cyclin B–cdc2 kinase is required to suppress DNA replication between meiosis I and meiosis II in starfish oocytes. *EMBO J.* **15,** 3590–3598.

Polyak, K., Kato, J., Solomon, M., Sherr, C. J., Massague, J., Roberts, J. M., and Koff, A. (1994). p27Kip1, a cyclin–cdk inhibitor, links transforming growth factor-B and contact inhibition to cell cycle arrest. *Genes Dev.* **8,** 9–22.

Richter, J. D. (1996). Dynamics of poly (A) addition and removal during development. *In* "Translational Control" (J. W. B. Hershey, M. B., Mathews, and N. Sonenberg, Eds.), pp. 481–503. Cold Spring Harbor Laboratory Press, Plainview, NY.

Rosenthal, E. T., and Ruderman, J. V. (1987). Widespread changes in the translation and adenylation of maternal messenger RNAs following fertilisation of *Spisula* oocytes. *Dev. Biol.* **121**, 237–246.

Ruderman, J. V. (1993). MAP kinase and the activation of quiescent cells. *Curr. Opin. Cell Biol.* **5,** 207–213.

Ruderman, J. V., Luca, F., Shibuya, E., Gavin, K., Boulton, T., and Cobb, M. (1991). Control of the cell cycle in early embryos. *Cold Spring Harbor Symp. Quant. Biol.* **56,** 495–502.

Sadler, K. C., and Ruderman, J. V. (1998). Components of the signaling pathway linking the 1-methyladenine receptor to MPF activation and maturation in starfish oocytes. *Dev. Biol.* **197,** 25–38.

Sagata, N. (1997). What does mos do in oocytes and somatic cells? *BioEssays* **19,** 13–21.

Sheets, M. D., Wu, M., and Wickens, M. (1995). Polyadenylation of c-*mos* mRNA as a control point in *Xenopus* meiotic maturation. *Nature* **374,** 511–516.

Shibuya, E. K., Boulton, T. G., Cobb, M. H., and Ruderman, J. V. (1992). Activation of p42 MAP kinase and the release of oocytes from cell cycle arrest. *EMBO J.* **11,** 3963–3975.

Standart, N. (1992). Masking and unmasking of maternal mRNA. *Semin. Dev. Biol.* **3**, 367–379.

Standart, N., Dale, M., Stewart, E., and Hunt, T. (1990). Maternal mRNA from clam oocytes can be specifically unmasked in vitro by antisense RNA complementary to the 3′-untranslated region. *Genes Dev.* **4,** 2157–2168.

Stebbins-Boaz, B., Hake, L. E., and Richter, J. D. (1996). CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and c-*mos* mRNAs and is necessary for oocyte maturation in *Xenopus*. *EMBO J.* **15,** 2582–2592.

Tachibana, K., Machida, T., Nomura, Y., and Kishimoto, T. (1997). MAP kinase links the fertilisation signal transduction pathway to the G1/S-phase transition in starfish eggs. *EMBO J.* **16,** 4333–4339.

Taieb, F., Thibier, C., and Jessus, C. (1997). On cyclins, oocytes, and eggs. *Mol. Reprod. Dev.* **48,** 397–411.

Toyoshima, H., and Hunter, T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk kinase activity, is related to p21. *Cell* **78,** 67–74.

Turner, J., Minkoff, C., Martin, K., Misra, R., and Swenson, K. (1995). Oocyte activation and passage though the metaphase/anaphase transition of the meiotic cell cycle is blocked in clams by inhibitors of HMG-CoA reductase activity. *J. Cell Biol.* **128,** 1145–1162.

Walker, J., Dale, M., and Standart, N. (1996). Unmasking mRNA in clam oocytes: Role of phosphorylation of a 3′UTR masking element-binding protein at fertilisation. *Dev. Biol.* **173,** 292–305.

Walker, J., Minshall, N., Hake, L., Richter, J., and Standart, N. (1999). The clam 3′UTR masking element-binding protein p82 is a member of the CPEB family. *RNA* **5,** 14–26.

Wickens, M., Kimble, J., and Strickland, S. (1996). Translational control of developmental decisions. *In* "Translational Control" (J. W. B. Hershey, M. B. Mathews, and N. Sonenberg, Eds.), pp. 411–450. Cold Spring Harbor Laboratory Press, Plainview, NY.

Winkler, M. M., Steinhardt, R. A., Grainger, J. L., and Minning, L. (1980). Dual ionic controls for the activation of protein synthesis at fertilisation. *Nature* **287,** 558–560.

Winkler, M. M., and Steinhardt, R. A. (1981). Activation of protein synthesis in a sea urchin cell-free system. *Dev. Biol.* **16,** 533–535.

Received for publication October 29, 1998
Revised February 16, 1999
Accepted February 17, 1999